ON THE NATURE OF STRUCTURES CHARACTERISTIC OF CNIDOSPORIDIAN SPORES[1]

By

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INTRODUCTION

Although our knowledge of the morphology and development of Cnidosporidia has recently been greatly increased, little is known about the chemical nature of the different parts which compose the spores. In the study of artificial cultivation of Cnidosporidia, it becomes necessary to obtain a definite view regarding this point. Unfortunately the majority of opinions advanced by several authors are not accompanied by any definite experimental data. This is possibly due to the fact that because the organisms have never been found in direct contact with higher vertebrates as their parasites, they have not interested such a large number of investigators as did other parasitic Protozoa which are directly responsible for serious diseases among mammals, and that the number of organisms found at one time is not generally large so that only little is left when the study of their morphology and development is completed.

Erdmann (1917:317–318) has recently expressed a view that the polar filaments of the spore of *Chloromyxum leydigi* were probably composed of glycogen and plastin, and further suggested, in referring to my paper on the polar filament of the spore of *Nosema bombycis* (Kudo, 1913), that her method might be useful for the study of the nature of polar filaments of the Microsporidian.

I have been working for some time on the subject by using several species of Microsporidia and Myxosporidia. The results thus far obtained in regard to the polar filaments are entirely different from those of Erdmann's. Besides, as I believe the results of observations upon the nature of spore membrane and the so-called iodinophilous vacuole seem to be more or less interesting, they are summarized and presented in the following pages.

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[1] Contribution from the Zoological Laboratory of the University of Illinois, No. 182.

[2] I am greatly indebted to Professor Henry B. Ward who kindly placed the material at my disposal.

MATERIAL AND METHODS

For the study of spore membrane of Myxosporidia, *Henneguya salminicola* Ward[2] was chosen. This species, as was described by Ward (1920), forms numerous large cysts in the host tissue. Moreover, it was not only obtainable in a very large number, but was also favorable for the study due to the presence of the posterior process characteristic of the genus. The Myxosporidian had been preserved in formol since 1914 together with the host tissue. The cysts were isolated, washed thoroughly with distilled water, and the cyst wall was punctured. The emulsion thus obtained was used to make numerous smears. For Microsporidia, spores of *Nosema bombycis* Nägeli and *Nosema apis* Zander were selected. Although they are much smaller than such a form as *Thelohania magna* Kudo (Kudo, 1920), the enormous number that could be procured favored their selection as representatives of Microsporidia. The infected silk-worm moths and the infected ventriculus and intestine of honey bees were emulsified with distilled water, and were used as stock emulsions.

The nature of the polar filaments was studied by using the fresh spores of *Myxobolus mesentericus* Kudo, *Mitraspora elongata* Kudo, *Leptotheca ohlmacheri* (Gurley) Labbé, *Nosema bombycis*, *Nosema apis* and *Thelohania magna*.

The following four species of the family Myxobolidae were used for the study of iodinophilous vacuole. They were fixed either in formol or sublimate alcohol as are indicated below: *Myxobolus discrepans* Kudo and *Myxobolus mesentericus* Kudo fixed in sublimate alcohol, and preserved in 95% alcohol; *Myxobolus aureatus* Ward and *Henneguya salminicola* Ward fixed and preserved in formol. Besides, the following five species from other genera were also studied for the comparison: *Wardia ovinocua* Kudo, *Mitraspora elongata* Kudo, *Leptotheca ohlmacheri* (Gurley) Labbé, *Chloromyxum wardi* Kudo and *Myxidium americanum* Kudo.

The experiments were conducted both in smears and section preparations. In former case, the smears were first allowed to dry before the application of chemicals or stains especially for the determination of spore membrane. Detailed descriptions of methods used will be given in the corresponding chapters.

THE SPORE MEMBRANE

The strong resistance of spores of *Nosema bombycis* against certain chemicals has long been known by the studies of Frei and Lebert (1856) and Haberlandt and Verson (1870), as the parasite is the cause of the well known pebrine disease of silk worms. These authors, however, attacked the problem with the aim of destroying the spores rather than the determination of its nature.

As to the spore membrane of Myxosporidia, Bütschli seems to be the first who studied the effect of concentrated sulphuric acid upon the spores of *Myxobolus mülleri* Bütschli. He (Bütschli, 1881: 634) states as follows: "Die Schalensubstanz besitzt eine recht ansehnliche Widerstands-fähigkeit gegen Reagentien; dennoch ist die Angabe Balbiani's, dass sie auch in erhitzten Mineralsäuren sich erhalte, nicht begründet. Erstmaliges Erhitzen in koncentrirter Schwefelsäure liess zwar die Schalen nur in ihre beiden Klappen zerfallen, zerstörte dagegen die sogleich zu erwähnenden Polkapseln völlig; nochmaliges Erhitzen bewirkt jedoch auch völlige Zerstörung der Schalen." Balbiani (1883:202) on the other hand states that the boiling sulphuric acid does not affect the Myxosporidian spore membrane. Gurley (1894:83) agreed with Bütschli, writing as follows: "This (shell) substance is thin, very transparent, insoluble in the strongest acids and alkalies in the cold, certainly in some, and probably in most species destroyed by (soluble in ?) concentrated sulphuric acid at its boiling temperature."

It is my opinion that the controversy of the results of experiments among these authors are probably due to the difference in the concentration of the acid used. Although none of the investigators mentioned their exact technique, it is almost certain that they added concentrated sulphuric acid to the emulsion of the spores they had. Consequently they did not observe the effect of the truly concentrated sulphuric acid, but that of more or less diluted acid which varied from a stronger concentration (Bütschli and Gurley) to a weaker one (Balbiani) according to the relative amounts of water and the acid. In order to avoid this error in the present experiments, the smears of spore emulsions were dried on the slides before they were subjected to the action of reagents.

None of the above mentioned three authors, however, has expressed an opinion in regard to the chemical nature of the membrane. It was Thélohan who advanced his observations concerning this point. Yet in his valuable work on Myxosporidia, Thélohan (1895:260) simply states as follows: "Je n'ai pas déterminé la nature chimique de la substance qui constitue l'enveloppe. Elle ne présente en tout cas aucun des charactères de la cellulose."

Although a large number of papers on Cnidosporidia has appeared lately, none touches this problem. Davis (1917:210) states that "surrounding the spore is a thin, tough, transparent membrane, the sporocyst, which is probably of a chitinoid nature.' He, however, does not give any experimental datum to support this statement. Auerbach (1910:17) in his monograph wrote negatively as follows: "Die chemische Zusammensetzung der Schale ist meines Wissens noch nicht sicher bekannt."

Thus the opinions of a few investigators regarding the chemical nature of the Cnidosporidian spore membrane may be summarized as follows: The spore membrane of Myxosporidia does not give a positive cellulose reaction, and seems to be of chitinoid nature.

The results of my experiments will be reported here.

a) Tests for albuminoid substances:—Spores of *Henneguya salminicola*, *Nosema bombycis* and *Nosema spis* are not affected by boiling potassium hydrate solution (35 per cent), and do not give any recognizable positive Millon's reaction. It may therefore be said that the spore membrane is not composed of albuminoid substances.

b) Tests for cellulose:—For the control of the cellulose tests, a filter paper was used.

1) Ammoniacal solution of copper oxide. Six fibers of the filter paper were taken out, and were mounted on two slides, each containing three fibers. To one distilled water was added, while to the other ammoniacal solution of copper oxide. Both were covered with cover glasses. The water emulsions of *Henneguya salminicola*, *Nosema bombycis* and *Nosema apis* were smeared on slides, were dried, and were treated in the same way as the fibers of filter paper. The preparations were kept in a moist chamber. The results of observations are as follows:

	Soon after the treatment	16 hours later	36 hours later
Cellulose with water	Outline of fibers sharp	No change	No change
Cellulose with ammon. solution of copper oxide	Outline of fibers less sharp	Outline faint	More invisible
Henneguya salm., *Nosema bombycis* and *N. apis* with water	Outline of spores sharp	No change	No change
Henn. salminicola, *Nosema apis*, and *N. bombycis* with amm. sol. of copper oxide	Outline of spores sharp	No change	No change

From the above, it is clear that the ammoniacal solution of copper oxide does not dissolve the spore membrane of *Henneguya salminicola*, *Nosema bombycis* and *Nosema apis*.

2) Lugol solution and sulphuric acid. Small pieces of filter paper were treated with Lugol solution, washed with distilled water, and were dried. They were mounted on slides in distilled water and in 50 per cent sulphuric acid respectively. Dried smears of spore emulsion of *Henneguya*

salminicola, *Nosema apis* and *Nosema bombycis* were treated in a similar way. The results of observations are as follows:

	Soon after preparation
Cellulose: water and H_2SO_4	No coloration
Cellulose: Lugol	Deep brown
Cellulose: Lugol and H_2SO_4	Violet
Henn. salm.: water and H_2SO_4	No coloration
Henn. salm.: Lugol	Slightly yellowish
Henn. salm.: Lugol and H_2SO_4	Slightly yellowish
Nosema apis and *bombycis:* water and H_2SO_4	No coloration
Nosema apis and *bombycis:* Lugol	Almost unstained
Nosema apis and *bombycis:* Lugol and H_2SO_4	Almost unstained

3) Zinc chloride-iodine-potassium iodide mixture. Fibers of a filter paper and dried smears of spores of *Henneguya salminicola*, *Nosema bombycis*, and *Nosema apis* were treated with the following mixture: Zincum chloratum pur. sicc. 20 gr., potassium iodide 6.5 gr., iodine 1.3 gr., and distilled water 10.5 cc. The results of observations are as follows:

	Soon after preparation	16 hours later
Cellulose with water	No staining	No staining
Cellulose with mixture	Violet blue	Violet blue
The spores with water	No staining	No staining
The spores with mixture	Slightly yellowish; iodinophilous vacuole of Henneguya brownish	No change

As will be seen from the above experiments, none of the cellulose tests gives positive reaction. It may therefore be stated as was remarked by Thélohan (1895) that the spore membrane of *Henneguya salminicola*, *Nosema bombycis* and *Nosema apis* is not of cellulose nature.

c) Tests for chitin:—For the control of chitin test, I have prepared chitin from the wings of honey bees.

1) Alkalies and acids. Dried smears of spores of *Henneguya salminicola*, *Nosema bombycis*, and *Nosema apis*, were treated with potassium hydrate solution and mineral acids. The results are as follows:

	Chitin	Spore membrane of *Henn. salminicola*	Spore membrane of *Nosema apis and Nosema bombycis*
Boiling KOH (35%)	Insoluble	Insoluble	Insoluble
Boiling dilute HNO_3	Soluble	Spore becomes more or less swollen; contents attacked	Spore becomes greatly swollen and hardly visible
Boiling conc. HNO^3	Soluble	Spore becomes larger; spore membrane of uniform thickness; less refractive	Spore becomes extremely enlarged; invisible.
Conc. HCl (room temp.)	Soluble	Slightly soluble; outline irregular	Spore enlarged and invisble
Boiling conc. HCl	Soluble	Attacked	Disintegrates rapidly
Dilute H_2SO_4	Insoluble	Insoluble	Insoluble
Conc. H_2SO_4 (room temp)	Soluble	Membrane becomes thinner; outline irregular; valves split; caudal filament broken	Greatly enlarged and invisible
Boiling conc. H_2SO_4	Soluble	Completely dissolved	Completely dissolved

2) Zinc chloride and Lugol solution. The spore emulsions of *Henneguya salminicola*, *Nosema bombycis* and *Nosema apis* were mixed with potassium hydrate solution (35 per cent), and washed thoroughly by centrifugation. After being partly dried, 1 cc. of a mixture of 33⅓ per cent aqueous solution of zinc chloride (10 cc.) and of strong Lugol solution (5 drops) was added, and observed. The results follow

	Color reaction
Chitin	Dark brown
Henneguya salminicola	Spore membrane very slightly yellowish
Nosema bombycis and *Nosema apis*	Spore membrane unstained

3) Potassium iodide and sulphuric acid. Chitin and dried smears of *Henneguya salminicola*, *Nosema bombycis* and *Nosema apis* were boiled at

160°C with potassium hydrate solution (35 per cent) for thirty minutes, washed thoroughly with 90 per cent alcohol, and then with distilled water. They were then treated with a weak solution of potassium iodide which had been acidified with sulphuric acid, and were examined. The results are as follows:

	Color reaction
Chitin	Bluish violet
Henneguya salminicola	No visible staining of the membrane
Nosema bombycis and *Nosema apis*	No visible staining of the membrane

As will be seen from the above experiments, the staining reaction gives very ambiguous results. On the other hand, the effect of mineral acids upon the spore membrane seems to be decisive. The spore membrane of *Nosema bombycis* and *Nosema apis* behaves very much like chitin under the influence of mineral acids, while that of *Henneguya salminicola* is more or less different in this respect.

The Polar Filament

Thélohan (1890:207) expressed an opinion that the substance composing the wall of the polar capsule was identical with that composing the spore membrane, as both stained in the same way with safranin. This view probably led Minchin (1912:399) to state that "a polar capsule is a hollow, pearshaped body with a tough envelope, probably chitinoid in nature. . . . Coiled up within the capsule is a delicate filament, often of great length, probably of the same nature as the capsule, and continuous with it." Minchin does not give any evidence to support this view. Davis (1917:210) possibly referred to Minchin, although he did not make it clear, when he stated as follows: "Surrounding the capsule is a tough, refractive envelope, probably chitinous. . . . Coiled up within the capsule is a delicate filament, usually of comparatively great length, which is probably of the same material as the capsule."

Erdmann (1917:317), by studying the developing polar filament of *Chloromyxum leydigi*, came to the conclusion that the polar filament is composed of glycogen and plastin. She writes as follows: "Die vier Polkapseln füllen jetzt ihre Mutterzelle aus, die Plastinscheiben werden zu Plastinringen, die durch Glykogen verbunden sind. Der Polfaden ist entstanden. . . . Dagegen kann ich die Umwandlung des Chromatins and des Plasmas in eine stark färbbare Substanz, nach meinen Befunden, Glykogen, bestätigen." To support this view, Erdmann further remarked the difficulty in extruding the polar filament of spores of the species of the genus Chloromyxum as follows "Ausgestreckte Polfäden von Chloromyxumsporen sind kaum an Präparaten beobachtet. Thélohan (Taf. IX, Fig. 100c) bildet die Polfäden einer frischen Spore von *Chloromyxum quadratum* ab, Erdmann *Chloromyxum leydigi* mit kurzem Polfäden. Auerbach ist es nicht gelungen, bei *Chloromyxum dubium* sie zu zeigen. Lebzelter erwähnt sie nicht bei *Chloromyxum thymalli*. Durch eine von mir ausgeprobte Methode der Fixierung (100 Proz. Alkohol bis auf 40 Grad erhitzt) gelingt es leicht, die Polfäden zum Austreten zu bringen und zu fixieren. Glykogenfärbung nach Fixierung zeigt, dass der Polfaden aus Glykogen und einer plastinähnlichen Substanz zusammengesetzt ist (Taf. 14, Fig. 27)." I have, however, had no difficulty in causing the extrusion of the polar filament from the fresh spores of three out of five species of Chloromyxum which I have studied up to date. In *Chloromyxum misgurni* Kudo (Kudo 1916, Fig. 3e), *Chloromyxum fujitai* Kudo (drawings were omitted due to the lack of space) and *Chloromyxum trijugum* Kudo (Kudo 1919: Fig. 181), I have caused the filament extrusion. The other two species, *Chloromyxum catostomi* Kudo (Kudo 1919) and *Chloromyxum wardi* Kudo (Kudo 1919) were studied only in fixed specimens, and no attempt was made to cause the filament extrusion. Erdmann has probably studied a small number of spores.

Regarding the various methods which had been reported by several investigators as successful in extruding the polar filaments of various Cnidosporidian spores, I already summarized them in one of my papers (Kudo 1918). Of many methods which I have tried since that date, the following gave always the best results. For Myxosporidian spores, potassium hydrate solution or perhydrol will always bring out satisfactory results. This is especially true in the case of tissue infecting forms. When the spores are found in the gall bladder or urinary bladder, the best results are obtained by centrifuging the spore containing bile or urine followed by repeated washing with distilled water before the spores are subjected to the influence of the chemicals, although this is not of absolute necessity. Yet when the number of spores present in the bile or urine is very small, the treatment is favorable as the addition of potassium hydrate solution to the bile produces a large amount of precipitation which hinders the observation greatly. In most cases, no staining of the filament of Myxosporidian spores seems to be necessary, due to the favorable thickness and distinctness even in unstained state. For staining, Fontana's method of staining spirochoetes, or Giemsa's solution gives beautiful results. The latter has its advantage over the former in bringing out the differentiation of nucleus and sporoplasm beside the filament, although in some cases the filament does not take the stain for unknown reasons. For Microsporidia, mechanical pressure or perhydrol gives beautiful preparations of extruded filaments. As I did not describe the detail of the method used (Kudo 1913), I have recently described the exact technique for the application of mechanical pressure elsewhere (Kudo 1920).

Although Erdmann was apparently unaware of it, the filament extrusion under the effect of absolute alcohol had been described by Ohlmacher (1893) in the case of *Leptotheca ohlmacheri* (Gurley) Labbé. In the section preparations of kidneys of *Bufo lentiginosus* fixed with absolute alcohol, Ohlmacher saw a number of spores with extruded polar filaments. Ohlmacher was of the opinion that "it is, of course, evident that they (polar filaments) must have been thrown out from spores before the organisms were killed by the alcohol employed in fixing." According to my own observations on a large number of section preparations obtained from *Rana clamitans*, it is clear that in Ohlmacher's preparation, the fixation with absolute alcohol which caused vigorous shrinkage of the spore membrane and sporoplasm, was only responsible for the presence of spores with extruded polar filaments. It is certain that the absolute alcohol method of Erdmann is according to my comparative study on various methods far inferior in having incompleteness and irregularity in its action.

The results of my observations are as follows:

1) The effect of water upon the polar filament. To determine whether the extruded polar filaments of spores of *Nosema bombycis*, *Nosema apis*, *Myxobolus mesentericus* and *Leptotheca ohlmacheri* are soluble in water or not, fresh spores were subjected to mechanical pressure. After removing the coverglasses, the smears were covered with distilled water, and were kept in a moist chamber. The examinations were done under a dark field microscope, and also in Fontana preparations. The results were similar in four species, which are as follows:

	Results of Observations
Control: soon after the application of mechanical pressure	Extruded polar filaments
One day in water	Polar filaments unchanged
Two days in water	Polar filaments unchanged
Four days in water	Polar filaments unchanged
Eight days in water	Polar filaments unchanged

The experiments were repeated many times on other species than mentioned above, but always giving the same results. From these experiments it may be concluded that the polar filaments of the spores of Cnidosporidia mentioned above are insoluble in distilled water at room temperature.

2) The effect of filtered saliva upon the polar filaments. To determine whether the extruded polar filaments of spores of the species mentioned above are soluble in filtered saliva or not, fresh spores were pressed mechanically. A drop of filtered saliva was added to each smear, and the smears were kept in a moist chamber. The examinations were done as in the preceding experiments, and revealed the following results which were practically the same in the four species:

	Observations
Control: soon after the extrusion	Extruded polar filaments
10 minutes in saliva	Polar filaments unchanged
30 minutes in saliva	Polar filaments unchanged
1 hour in saliva	Polar filaments unchanged
3 hours in saliva	Polar filaments unchanged
16 hours in saliva	Polar filaments unchanged
32 hours in saliva	Polar filaments unchanged
3 days in saliva	Polar filaments unchanged
6 days in saliva	Polar filaments unchanged

From the experiments, it may be said that the extruded polar filaments of the spores of *Leptotheca ohlmacheri*, *Myxobolus mesentericus*, *Nosema bombycis* and *Nosema apis*, are insoluble in filtered saliva at room temperature.

3) Staining with Lugol solution. The extruded polar filaments of spores of *Nosema bombycis*, *Nosema apis*, *Thelohania magna*, *Leptotheca ohlmacheri* and *Myxobolus mesentericus* stain uniformly as light yellowish as their spore membrane by Lugol solution, and do not take any deeper color.

4) Staining after Best's method. The extruded polar filaments of spores of *Nosema bombycis*, *Nosema apis*, *Thelohania magna*, *Leptotheca*

ohlmacheri and *Myxobolus mesentericus* remain unstained by Best's method.

5) Staining after Lubarsch's method. The extruded polar filaments of spores of *Nosema bombycis*, *Nosema apis*, *Thelohania magna*, *Leptotheca ohlmacheri* and *Myxobolus mesentericus* stain uniformly slightly bluish-violet by Lubarsch's method. The polar capsules of the latter two species and the spore membranes frequently stain deep violet.

6) Staining by Löffler's method. The extruded polar filaments of spores of *Nosema bombycis* (Kudo, 1913), and of *Nosema apis*, *Thelohania magna*, *Leptotheca ohlmacheri* and *Chloromyxum trijugum* are stained deep violet by Löffler's method. The spore membrane is also stained in the same color.

7) Staining with Giemsa's stain. The extruded polar filament of *Nosema bombycis* (Kudo, 1916), and of *Nosema apis* and *Chloromyxum trijugum* (Kudo 1920, Fig. 181) have been stained in deep, dark red. The polar capsules of the latter species and the spore membrane also frequently stain the same color.

8) Staining with Fontana's mixture for staining spirochoetes. The extruded polar filaments of *Nosema bombycis*, *Nosema apis*, *Thelohania magna*, *Thelohania illinoisensis*, *Leptotheca ohlmacheri*, *Chloromyxum trijugum* stain in from yellowish to dark brown color by Fontana's method. The spore membrane takes the stain in the similar manner.

From these experiments, it is clear that the polar filaments of spores of various species of Cnidosporidia, which have been listed in the above, are not composed of glycogen as was thought by Erdmann in the case of *Chloromyxum leydigi*. The only means which led Erdmann to the already quoted conclusion regarding the chemical nature of the polar filament is the results of Lubarsch's staining. My experiments have shown clearly that while this staining brings out more or less bluish stained filaments, other tests for its glycogenous nature proved to be negative. The staining effect of Löffler's method is similar to that on the flagella of *Bacillus typhosus*, and that of Fontana's method is exactly the same as that on various spirochoetes.

As to its true nature, I am, however, still unable to determine. It has been noted by many investigators in numerous species of Myxosporidia, and by myself in *Leptotheca ohlmacheri* and *Thelohania magna* that the nucleus for the polar capsule becomes nebulous or diffused during the formation of the polar filament. The chromatic substance of the nucleus breaks up into numerous small granules and a large part of it unites with a peculiar substance or substances which become differentiated in the capsulogenous cell, first in a retort shape and then in rounded form. The polar filament is apparently formed from this mixture.

THE IODINOPHILOUS VACUOLE

In the mature spores of Myxosporidia belonging to the family Myxobolidae, there exists regularly a more or less conspicuous rounded space which is generally known as an iodinophilous vacuole because of its behavior toward iodine.

Müller (1841) seems to be the first to notice this peculiar structure and figured vacuoles in his drawings. Bütschli (1881:636) observed the vacuole in the spore of *Myxobolus mülleri* which had previously been seen by Müller, and designated it as a nucleus. He described the structure as follows: "Von besonderem Interesse erscheint das unzweifelhafte Vorhandensein eines Zellkerns in der plasmatischen Inhaltsmasse der Sporen. Häufig ist dieser Kern schon in frischem Zustand ohne Weiteres als kreisförmiger bis ovaler, heller Fleck recht deutlich sichtbar (Fig. 1n). Besser tritt er jedoch nach Behandlung mit verdünnter Essigsäure oder Jodtinktur hervor und zeigt dann eine dunkle, etwas granulirt erscheinende Hülle (Fig. 2n) und eine Anzahl ziemlich blasser Granula, welche durch den Inhalt zerstreut sind. Leider setzten sich dem Versuch, den Kern zu färben, sehr energische Hindernisse entgegen, da das Färbungsmittel nicht in die Sporenschale eindringt; jedoch kann dieser Umstand nicht gegen Kernnatur des fraglichen Gebildes angeführt werden, da auch das Plasma der Färbung widerstand. Dennoch beobachtete ich einige Fälle deutlicher Kernfärbung bei Anwendung von Alaunkarmin."

That Bütschli's view was not correct was shown by Thélohan (1899: 919–920) who studied the structure more closely with the following statements: "Si, en effet, on traite ces spores par différentes réactifs, on acquiert bientôt la certitude que la tache claire observée par M. Bütschli, et décrite et figurée par lui comme un noyau, est en réalité une formation d'ordre tout différent. Peu visible à l'état frais, à-cause de la transparence du protoplasma, elle apparaît plus nettement par l'action de l'alcool, des acides acétique, azotique, osmique ou du nitrate d'argent à 2 pour 100. On la voit alors entourée par le reste de la masse plasmique, qui, coagulée sous l'influence de ces liquides, se distingue par son aspect finement granuleux et sa moindre réfringence. Elle a tous les caractères d'une vacuole creusée au sein de cette mass et remplie d'une substance particulière, remarquable par sa résistance aux réactifs colorants caractéristiques de la substance nucléaire. Seul l'iode se-fixe sur elle, et, tandis que, sous son influence, le reste de la spore prend une coloration d'un jaune pâle, on voit cette vésicule devenir d'un rouge brunâtre qui rappelle absolument la teinte que prend la matière glycogène par l'action de ce réactif. Comme celle-ci, cette substance est insoluble dans l'alcool et grade sa réaction vis-à-vis de l'iode dans les spores conservées dans ce liquide. Comme elle encore, elle est soluble dans les alcalis. Les acides la modifient, et après leur action elle ne se colore plus. Toutefois, je n'ai pu, dans ces circonstances, obtenir la réduction de la liqueur cupro-potassique." Gurley (1894:209) is in entire accord with the observations of Thélohan quoted above.

Keysselitz (1908:264) is the only other investigator who studied rather closely the structure under consideration. He remarks as follows: "Die Vacuole hat eine rundliche Form; ihre Grösse ist nicht ganz konstant. An der lebenfrischen Spore kann man sie nicht oder kaum bemerken. Nach Behandlung mit Argentum nitricum, Alkohol, Osmiumsaure (Vgl. Thélohan) Aqua destillata, gewöhnlichem Wasser (bei einzelner Sporen) beim Erhitzen sowie beim Antrocknen tritt sie deutlicher als heller Bezirk hervor. Sie ist gegen das umgebende Plasma nicht durch eine deutliche Membran abgesetzt. Beim Zusatz von wässeriger oder alkoholischer Jodlösung, färbt sich ihr Inhalt mahoganibraun, eine Reaktion, die für die Sporen der Myxobolen specifisch zu sein scheint. Er erscheint dann zuweilen fast homogen, häufiger bemerkt man verschwommene dunklere und hellere Flecke verschiedener Grösse und Form. Der Inhalt scheint mir eine zähflüssige Substanz zu sein, die in der Zelle gleichsam suspendiert ist. In konservierten, mit Farbstoffen behandelten Sporen tingiert sich die Vacuole nicht. Sie imponiert als heller Fleck in der Copula. Durch das Jod wird in der Regel auch in den zwischen den Polkapseln befindlichen Räume ein kleiner nicht scharf umgrenzter Bezirk mahoganibraun gefärbt." Auerbach (1910:16) simply states that "die Vacuole färbt sich bei Zusatz von Jodtinktur braun."

To summarize the views advanced by previous authors, the so-called iodinophilous vacuole is stained with iodine mixtures, and therefore is of glycogenous nature.

My observations gave the following results.

1) The effect of distilled water upon the vacuole. In order to determine whether the vacuole is affected by distilled water in partly exposed conditions or not, fresh spores of *Myxobolus mesentericus* and *Henneguya mictospora* were crushed under the cover glass, and were kept in distilled water. After six hours, the smears were treated with Lugol solution, which gave the following results on examination:

	Observations
Control spores without being pressed	Typical coloration of the vacuole
Spores crushed	No visible staining of vacuole-like structure

The experiments show that the contents of the vacuole disappear when placed in contact with distilled water.

2) Treatment with Lugol solution. Smears and section preparations of *Henneguya salminicola, Myxobolus aureatus, Myxobolus discrepans* and *Henneguya mictospora* were treated with Lugol solution. The vacuoles stained in brownish orange which on warming disappeared.

3) Staining by Lubarsch's method. Section preparations of *Henneguya salminicola* were stained in Lubarsch's mixture, the iodinophilous vacuole, the polar capsules as well as spore membrane were stained in deep bluish violet.

4) Staining by Best's method. Section preparations of *Henneguya salminicola* were stained by Best's method. The vacuole took a faint pink color.

5) Staining with Delafield's haematoxylin and Lugol solution. Section preparations and smears of *Henneguya salminicola* and *Myxobolus mesentericus* were first stained with Delafield's haematoxylin. After being washed thoroughly, they were mounted in gum and Lugol mixture. The iodinophilous vacuole stained in reddish brown.

From the experiments mentioned above, it is certain that the so-called iodinophilous vacuole of spores of the family Myxobolidae contains a substance similar to glycogen in characters.

In the section preparations of the cysts of *Henneguya salminicola*, one sees the appearance of the vacuole as the spore formation proceeds. The glycogenous substance in the sporoplasm remains inconspicuous while the spore is in the pansporoblast, although one can trace the gradual concentration of the substance in it. When the spore matures and separates itself from the other spore, the vacuole becomes sharply outlined. The vacuole reaches its maximum size when the spore is completely formed.

No particular body that corresponds to the iodinophilous vacuole was found in the spores of species belonging to families other than Myxobolidae, although I have tested several species repeatedly.

It is generally understood without any experimental evidence that the glycogen occuring in the spores of the family Myxobolidae is probably used for the future development of the sporoplasm. Then it is strange to notice the fact that the spores of other families which are essentially the same in habitat and in many other respects, do not contain the glycogen in such a conspicuous way as in this particular family. The majority of species belonging to the family Myxobolidae attack the tissue of the host, yet some species of the genera Chloromyxum, Myxidium and Sphaerospora and all the species of the family Myxosomatidae which do not show any iodinophilous vacuole in the spore at any stage of its development, inhabit also the tissue of the host. Therefore the occurrence of the iodinophilous vacuole does not seem to be correlated with the tissue infesting characters of the Myxosporidia, as was suggested by Gurley (1894).

Summary

1) The spore membrane of *Nosema apis* and *Nosema bombycis*, taken as representatives of Microsporidia, is proved to be composed of a substance similar to chitin in its chemical reaction.

2) The spore membrane of *Henneguya salminicola*, taken as a representative of Myxosporidia, is proved to be composed of a substance, the chemical reactions of which are less similar to those of chitin compared with the microsporidian spore membrane.

3) The polar filaments of cnidosporidian spores are not composed of glycogen as was suggested by Erdmann. They are formed by the mixture of a part of the nucleus and a substance differentiated in the capsulogenous cell.

4) A review of the methods which cause the filament extrusion in Cnidosporidian spores is presented.

5) The so-called iodinophilous vacuole of the spores of the family Myxobolidae contains a substance, the chemical reactions of which are similar to those of glycogen.

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